

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

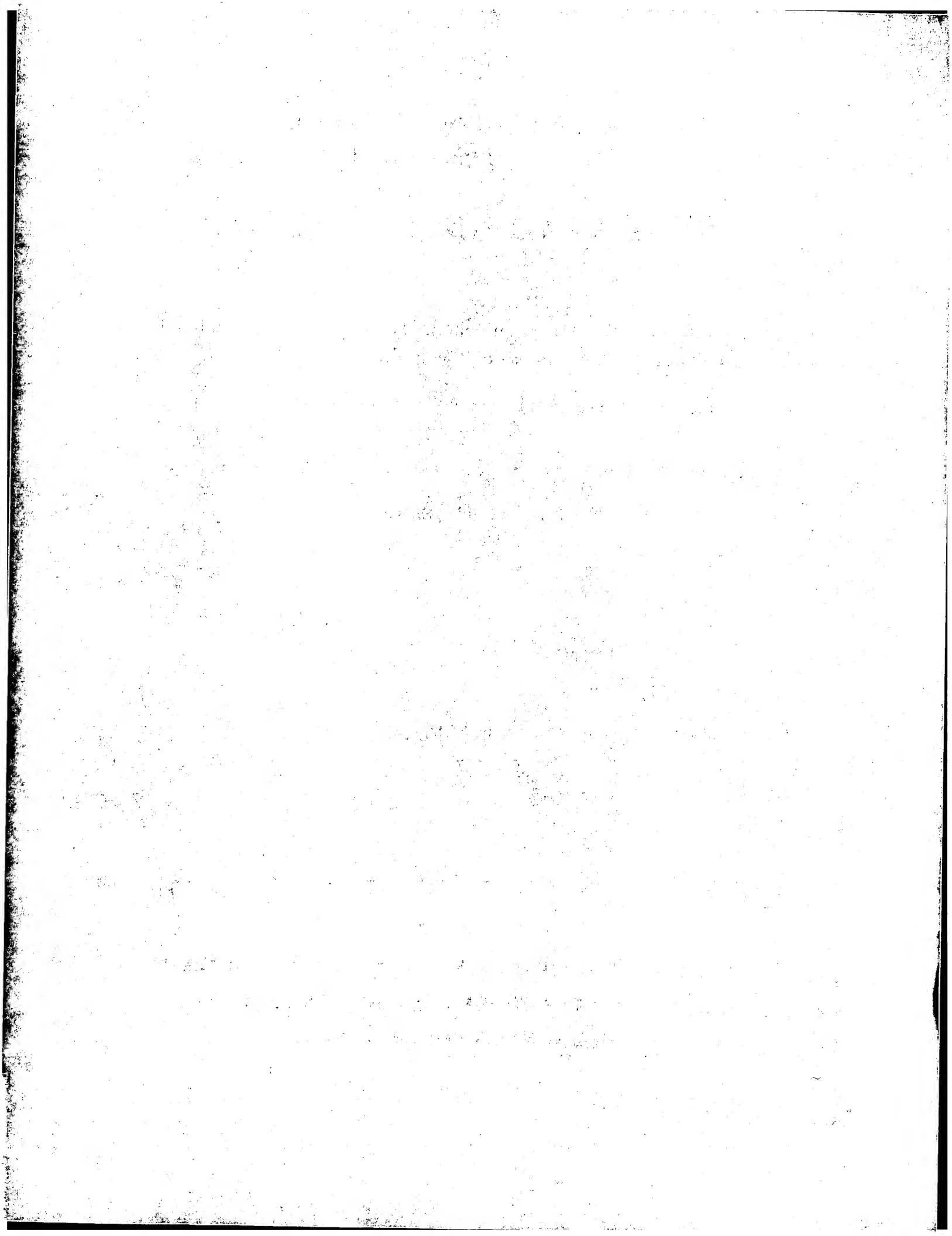
Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**





(21) (A1) 2,240,609
(22) 1998/08/11
(43) 1999/10/14

(72) NICOLAIDES, Nicholas, US

(72) VOGELSTEIN, Bert, US ,

(72) KINZLER, Kenneth W., US

(71) THE JOHNS HOPKINS UNIVERSITY, US

(51) Int.Cl. ⁶ C12N 5/10, C12N 15/85, C12Q 1/68, G01N 33/68, A01K 67/027

(30) 1998/04/14 (09/059,461) US

(54) **METHODE DE CREATION D'ORGANISMES
HYPERMUTABLES**

(54) **A METHOD FOR GENERATING HYPERMUTABLE
ORGANISMS**

(57) Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation.



Industrie Canada Industry Canada

A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

This invention was made using a U.S. government grant from the NIH (CA43460). Therefore, the U.S. government retains certain rights to the invention.

5 TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of mismatch repair genes. In particular it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

Within the past four years, the genetic cause of the Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome II, has been ascertained for the majority of kindreds affected with the disease (13). The molecular basis of HNPCC involves genetic instability resulting from defective mismatch repair (MMR). To date, six genes have been identified in humans that encode for proteins and appear to participate in the MMR process, including the *mutS* homologs *GTBP*, *hMSH2*, and *hMSH3* and the *mutL* homologs *hMLH1*, *hPMS1*, and *hPMS2* (2,7,11,17,20,21,22, 24). Germline mutations in four of these genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified in HNPCC

- 2 -

kindreds (2,11,13,17,24). Though the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to MMR abnormalities (14). Microsatellite instability is therefore a useful indicator of defective MMR. In addition to its 5 occurrence in virtually all tumors arising in HNPCC patients, Microsatellite instability is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (27).

HNPCC is inherited in an autosomal dominant fashion, so that the 10 normal cells of affected family members contain one mutant allele of the relevant MMR gene (inherited from an affected parent) and one wild-type allele (inherited from the unaffected parent). During the early stages of tumor development, however, the wild-type allele is inactivated through a somatic mutation, leaving the cell with no functional MMR gene and resulting in a profound defect in MMR activity. Because a somatic mutation 15 in addition to a germ-line mutation is required to generate defective MMR in the tumor cells, this mechanism is generally referred to as one involving "two hits," analogous to the biallelic inactivation of tumor suppressor genes that initiate other hereditary cancers (11,13,25). In line with this two-hit mechanism, the non-neoplastic cells of HNPCC patients generally retain 20 near normal levels of MMR activity due to the presence of the wild-type allele.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for rendering cells hypermutable.

25 It is another object of the present invention to provide genetically altered cell lines.

It is yet another object of the present invention to provide a method to produce transgenic animals that are hypermutable.

30 It is also an object of the present invention to provide genetically altered transgenic animals.

- 3 -

It is a further object of the invention to provide a method of mutating a gene of interest in a cell.

Yet another object of the invention is to provide a method of mutating a gene of interest in an animal.

5 These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making a hypermutable cell is provided. A polynucleotide encoding a dominant negative allele of a mismatch repair gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene.

10 In another embodiment of the invention, an isolated hypermutable cell is provided. The cell comprises a dominant negative allele of a mismatch repair gene.

15 In another embodiment of the invention, a hypermutable transgenic animal is provided. The animal comprises a dominant negative allele of a mismatch repair gene.

20 In another embodiment of the invention, a method is provided for introducing a mutation into a gene of interest. A polynucleotide encoding a dominant negative allele of a mismatch repair gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene. The cell further comprises a gene of interest. The cell is grown. The cell is tested to determine whether the gene of interest harbors a mutation.

25 In another embodiment of the invention, a method is provided for generating a mutation in a gene of interest. A transgenic animal comprising a polynucleotide encoding a dominant negative allele of a mismatch repair gene is grown. The animal comprises a gene of interest. The animal is tested to determine whether the gene of interest harbors a mutation.

30 These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations.

- 4 -

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Diagrams of *PMS2* expression vectors (Fig. 1A) and pCAR reporters (Fig. 1B).

5

Figure 2. SH cells co-transfected with pCAR reporters and *PMS2* expression vectors after 17 days of drug selection. (Fig. 2A) Western blots of lysates from untransfected SH cells (lane 1) or SH cells transfected with *PMS2-NOT* (lane 2) or *PMS2-WT* (lane 3). The arrow indicates the 110 kD protein expected for hPMS2. (Fig. 2B) Western blots of lysates from untransfected SH cells (lane 1) or SH cells transfected with *PMS2-NOT* (lane 2) or *PMS2-134* (lane 3). The arrow indicates the 14 kD protein expected for hPMS-134. Both A and B were probed with an antibody generated against the N-terminus of hPMS2. The upper polypeptides in A and the lower polypeptides in B represent cross-reactive hamster proteins. (Fig. 2C) β -galactosidase activity in lysates derived from SH cells co-transfected with *PMS2-NOT* (lane 1), *PMS2-WT* (lane 2), or *PMS2-134* (lane 3) plus reporter plasmid. Relative β -galactosidase activities are defined as the ratio of β -galactosidase activity in cells transfected with pCAR-OF compared to that in cells transfected with pCAR-IF; this normalization controlled for transfection efficiency and controlled for β -galactosidase activity in the cells expressing the various *PMS2* effector genes.

10

15

20

25

30

Figure 3. *In situ* β -galactosidase activity of pooled clones of SH cells stably transduced with the *PMS2-NOT* (Fig. 3A), *PMS2-WT* (Fig. 3B), or *PMS2-134* (Fig. 3C) expression vectors, then re-transfected with pCAR-OF reporter. After 17 days of drug selection, the colonies were pooled, cultured, and stained for β -galactosidase activity. A pooled culture of *PMS2-134* transduced SH cells expressing β -galactosidase from pCAR-OF

- 5 -

is visible in Fig. 3C. The level of expression is lower, as expected, than in SH cells transfected with the pCAR-IF reporter plasmid, shown as a positive control in Fig. 3D. Each of the fields illustrated is representative of that found in triplicate experiments.

5

10

15

20

25

30

Figure 4. Protein expression and β -galactosidase activity in stably transduced SH clones. (Fig. 4A) Western blots of lysates from clones stably transduced with PMS2-NOT (lanes 1-3) or PMS2-WT (lanes 4-6). (Fig. 4B) Western blots of lysates from clones stably transduced with PMS2-NOT (lanes 1-3) or PMS2-134 (lanes 4-6). (The arrows indicate the polypeptide of the appropriate molecular weight. The upper (Fig. 4A) and lower (Fig. 4B) molecular weight polypeptides are nonspecific proteins. (Fig. 4C) The clones expressing PMS2-NOT (lane 1A-3A), PMS2-WT (lanes 1B-3B), or PMS2-134 (lanes 1C-3C) were transduced with pCAR-OF or pCAR-IF reporter plasmids and multiple subclones selected in hygromycin plus geneticin were harvested 17 days later and assayed for β -galactosidase activity. Relative β -galactosidase activities are defined as the ratio of β -galactosidase activity in cells transduced with pCAR-OF compared to that in cells transduced with pCAR-IF.

Figure 5. Immunoprecipitation of *in vitro* translated hPMS2 and hMLH1 proteins. (Fig. 5A) Labelled (indicated by an asterisk) or unlabelled proteins were incubated with an antibody to the C-terminus of hPMS2 in lanes 1-3 and to hMLH1 in lanes 4-6. Lane 7 contains a nonprogrammed reticulocyte lysate. The PMS-135 contains codons 135-862 of hPMS2. The major translation products of hPMS2 and hMLH1 are indicated. (Fig. 5B) Labelled hPMS-134 (containing codons 1-134 of hPMS2) was incubated in the presence or absence of unlabelled hMLH1 plus an antibody to hMLH1 (lanes 1 and 2, respectively). Lane 3 contains lysate from a nonprogrammed reticulolysate. (Fig. 5C) Labelled proteins were incubated with an antibody to the N-terminus of hPMS2. Lane 6 contains a nonprogrammed

- 6 -

reticulocyte lysate. In both Fig. 5A and Fig. 5B, autoradiographs of immunoprecipitated products are shown.

Figure 6. Complementation of MMR activity in transduced SH cells.
5 Lysates from pooled clones stably transduced with *PMS2-NOT*, *PMS2-WT*, or *PMS2-134* were complemented with purified *MutS α* or *MutL α* MMR components using the 5'G/T heteroduplex substrate. The values are presented as the percentage of repair activity in each case compared to that in lysates complemented with both purified *MutL α* and *MutS α* components to normalize for repair efficiency in the different lysate backgrounds. The values shown represent the average of at least three different determinations.
10

DETAILED DESCRIPTION OF THE INVENTION

15 The inventors have discovered a method for developing hypermutable cells and animals by taking advantage of newly discovered alleles of human mismatch repair genes. Dominant negative alleles of such genes, when introduced into cells or transgenic animals, increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. Hypermutable cells or 20 animals can then be utilized to develop new mutations in a gene of interest.

25 The process of mismatch repair, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A mismatch repair gene is a gene that encodes one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a mismatch repair complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base which is complementary to the older DNA strand.
30 In this way, cells eliminate many mutations which occur as a result of mistakes in DNA replication.

- 7 -

5

10

20

25

30

Dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134. The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele which produces such effect can be used in this invention.

Dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Such alleles can be identified by screening cells for defective mismatch repair activity. The cells may be mutagenized or not. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic DNA, cDNA, or mRNA from any cell encoding a mismatch repair protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other mismatch repair genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

A cell or an animal into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation

- 8 -

of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal.

According to one aspect of the invention, a polynucleotide encoding a dominant negative form of a mismatch repair protein is introduced into a cell or a transgenic animal. The gene can be any dominant negative allele 5 encoding a protein which is part of a mismatch repair complex, for example, *PMS2*, *PMS1*, *MLH1*, or *MSH2*. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in 10 the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide. The polynucleotide can be introduced into the cell by transfection.

Transfection is any process whereby a polynucleotide is introduced 15 into a cell. The process of transfection can be carried out in a living animal, e.g., using a vector for gene therapy, or it can be carried out *in vitro*, e.g., using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa or yeast.

20 In general, transfection will be carried out using a suspension of cells, or a single cell, but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed 25 in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the mismatch repair gene, 30 the cell can be grown and reproduced in culture. If the transfection is stable,

- 9 -

such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

5

An isolated cell is a cell obtained from a tissue of humans or animals by mechanically separating out individual cells and transferring them to a suitable cell culture medium, either with or without pretreatment of the tissue with enzymes, e.g., collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or may be derived from suspensions of single-celled organisms.

10

A polynucleotide encoding a dominant negative form of a mismatch repair protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, e.g., cows, pigs, sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, e.g., cows, pigs, or goats that express a recombinant protein in their milk; or experimental animals for research or product testing, e.g., mice, rats, hamsters, guinea pigs, rabbits, etc.

20

15

Any method for making transgenic animals known in the art can be used. According to one process of producing a transgenic animal, the polynucleotide is injected into a fertilized egg of the animal and the injected egg is placed into a pseudo-pregnant female. The egg develops into a mature animal in which the polynucleotide is incorporated and expressed. The fertilized egg is produced *in vitro* from the egg and sperm of donor animals of the same species as the pseudo-pregnant female, who is prepared by hormone treatments to receive the fertilized egg and become pregnant. An alternative method for producing transgenic animals involves introducing the polynucleotide into embryonic cells by injection or transfection and reintroducing the embryonic cells into the developing embryo. With this

25

30

- 10 -

method, however, if the polynucleotide is not incorporated into germline cells, the gene will not be passed on to the progeny. Therefore, a transgenic animal produced by this method must be evaluated to determine whether the gene is incorporated into germ cells of the animal. Once transgenic animals are produced, they can be grown to reproductive age, when they can be mated to produce and maintain a colony of transgenic animals.

5

Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic animal or introduced into the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers.

10

15 Mutations can be detected by analyzing for alterations in the

genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening the phenotype of the gene. A mutant phenotype can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest.

20

25

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

30

EXAMPLE 1: *hPMS2-134* Encodes a Dominant Negative Mismatch Repair Protein.

A profound defect in MMR was found in the normal cells of two HNPCC patients. That this defect was operative *in vivo* was demonstrated by the widespread presence of microsatellite instability in non-neoplastic cells of such patients. One of the two patients had a germ-line truncating mutation of the *hPMS2* gene at codon 134 (the *hPMS2-134* mutation), while the other patient had a small germ-line deletion within the *hMLH1* gene (26). These data thus contradicted the two-hit model generally believed to explain the biochemical and biological features of HNPCC patients. The basis for this MMR deficiency in the normal cells of these patients was unclear, and several potential explanations were offered. For example, it was possible that the second allele of the relevant MMR gene was inactivated in the germ-line of these patients through an undiscovered mechanism, or that unknown mutations of other genes involved in the MMR process were present that cooperated with the known germ-line mutation. It is clear from knock-out experiments in mice that MMR-deficiency is compatible with normal growth and development, supporting these possibilities (1,3,6). Alternatively, it was possible that the mutant alleles exerted a dominant negative effect, resulting in MMR deficiency even in the presence of the wild-type allele of the corresponding MMR gene and all other genes involved in the MMR process. To distinguish between these possibilities, we expressed the truncated polypeptide encoded by the *hPMS2-134* mutation in an MMR proficient cell line and analyzed its effect on the cell's MMR activity. The results showed that this mutant could indeed exert a dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency.

The MMR proficient Syrian hamster TK-ts13 cell line (hereafter called SH cells) was cotransfected with various *hPMS2* expression plasmids plus reporter constructs for assessing MMR activity. The *hPMS2* expression plasmids contained the normal *hPMS2* gene product or the

- 12 -

truncated *hPMS2* gene identified in the patient described above (PMS2-WT and PMS2-134, respectively, Fig. 1A). An "empty" vector devoid of *hPMS2* sequences (PMS2-NOT, Fig. 1A) served as an additional control. The reporter construct pCAR-OF (out of frame) contained a hygromycin 5 resistance gene plus a β -galactosidase gene containing a 29 bp out-of-frame poly-CA tract at the 5' end of its coding region. The reporter construct pCAR-IF (in frame) was identical except that the poly-CA tract was 27 bp and therefore did not disrupt the β -galactosidase reading frame (Fig. 1B). The pCAR-OF reporter would not generate β -galactosidase activity unless a 10 frame-restoring mutation (*i.e.*, insertion or deletion) arose following transfection.

Three different transfection schemes were used to evaluate the 15 effects of the *PMS2-134* mutation on SH cells. In the first scheme, the expression vectors plus the reporters were co-transfected together. Pools containing greater than 100 clones were generated following 20 selection with hygromycin for 17 days and harvested for Western blot and β -galactosidase assays. SH cells transduced with *PMS2-WT* and *PMS2-134* synthesized polypeptides of the expected size, as assessed with anti-*hPMS2* antibodies on Western blots (Fig. 2A and 2B). As 25 expected, virtually no β -galactosidase activity was observed in SH cells transfected with the pCAR-OF reporter plus *PMS2-NOT* (Fig. 2C). However, SH cells transfected with *PMS2-134* expressed considerable β -galactosidase activity, significantly more than those transfected with *PMS2-WT* (Fig. 2C). These results suggested that 30 the truncated polypeptide encoded by the *PMS2-134* construct perturbs the endogenous MMR machinery, resulting in deletions or insertions that restored the reading frame. The exact nature of these presumed deletions or insertions could not be assessed, as multiple copies of the reporter constructs were transduced under our conditions, and the wild type β -galactosidase sequence was in great

- 13 -

excess over the expected mutants, precluding their demonstration by direct sequencing.

In the second scheme, SH cells were co-transfected with each of the PMS2 expression vectors plus the hygromycin-resistance plasmid pLHL4. Hygromycin resistant cultures containing greater than 100 clones were pooled and expanded. These cultures were then co-transfected with pCAR-IF or pCAR-OF reporters plus a separate plasmid allowing geneticin selection. Two weeks later, the pooled cells, each containing more than 100 colonies resistant to both hygromycin and geneticin, were stained with X-gal to assess β -galactosidase activity. As shown in Figure 3, the cultures transfected with *PMS2-134* (panel C) contained many blue cells, while virtually no cells were blue in the cultures transfected with *PMS2-NOT* or *PMS2-WT* (panels A and B, respectively). In each case, transfection efficiency was controlled by parallel transfections using pCAR-IF which also served as a control for β -galactosidase activity of cells expressing the various PMS2 effector genes, which resulted in similar β -galactosidase expression levels in all cases (example in Fig. 3D). Increases in β -galactosidase activity after *PMS2-134* transfection compared to *PMS2-WT* transfection were also observed when a similar experimental protocol was applied to the MMR-proficient human embryonic kidney cell line 293. These cells were cotransfected with the pCAR-OF plus the various PMS2 effector plasmids and selected for 17 days in hygromycin. At day 17, colonies were stained with X-gal to assess β -galactosidase activity and scored for β -galactosidase expressing cells. As shown in Table 1, only those cells expressing the PMS2-134 polypeptide expressed a detectable β -galactosidase activity. These data demonstrate a similar dominant negative effect of the hPMS2-134 protein in both rodent and human systems and validate the utility of the rodent system in these studies.

- 14 -

5

10

15

20

In the third scheme, SH cells were transfected with each of the *PMS2* expression vectors as described for the second scheme, but individual clones, rather than pooled clones, were expanded following drug selection. Of twenty clones transfected with *PMS2-WT*, five were shown to express readily detectable levels of full-length PMS2 proteins (examples in Fig. 4A, lanes 4-6). Similar analyses of twenty *PMS2-134* clones revealed four clones which expressed truncated PMS2 polypeptides of the expected size (examples in Figure 4B, lanes 4-6). Three clones expressing full-length or truncated PMS2 proteins, as well as three randomly selected clones from *PMS2-NOT* transfected cells (Figure 4A and 4B, lanes 1-3) were chosen for further analysis. The individual clones were tested for β -galactosidase activity following co-transfection with pCAR-OF plus the pNTK plasmid, as described above for the pooled clones. As shown in Figure 4C, each of the three clones (lanes 3A-3C) expressing the truncated hPMS2 polypeptide yielded much higher β -galactosidase activities following transfection with pCAR-OF than did the clones expressing the full-length hPMS2 protein (lanes 2A-2C) or no hPMS2 protein (lanes 1A-1C).

- 15 -

Table 1. β -galactosidase expression of 293 clones transfected with pCAR-OF reporter construct plus PMS2 effector plasmids. 293 cells were cotransfected with the pCAR-OF β -galactosidase reporter plasmid plus the *PMS2-NOT*, *-WT*, or *-I34* effector plasmids.

5 Transfected cells were selected in hygromycin for 17 days and stained with x-gal for β -galactosidase activity (blue colored cells). The results below represent the mean +/- standard deviation of triplicate experiments.

10	<u>Sample</u>	<u>Blue colonies</u>	<u>White colonies</u>
	<i>PMS2-NOT</i>	0 +/- 0	17 +/- 2.7
	<i>PMS2-WT</i>	0 +/- 0	18 +/- 4.0
	<i>PMS2-I34</i>	15 +/- 2.1	6 +/- 2.1

15

- 16 -

5

Plasmids. The full-length wild-type *hPMS2* cDNA was obtained from a human Hela cDNA library as described (18). An *hPMS2* cDNA containing a termination codon at amino acid 134 was obtained via RT-PCR from the patient in which the mutation was discovered (9). The cDNA fragments were cloned into the BamHI site into the pSG5 vector, which contains an SV40 promoter followed by an SV40 polyadenylation signal (8). The pCAR reporter vectors described in Fig. 1 were constructed as described in ref. 21 and 25.

10

15

Cell lines and transfection. Syrian Hamster fibroblast Tk^{ts}13 cells were obtained from ATCC and cultured as described (15). Stably transfected cell lines expressing *hPMS2* were created by cotransfection of the *PMS2* expression vectors and the pLHL4 plasmid encoding the hygromycin resistance gene at a ratio of 3:1 (pCAR:pLHL4) and selected with hygromycin. Stably transfected cell lines containing pCAR reporters were generated by co-transfection of pCAR vectors together with either pNTK plasmid encoding the neomycin resistance plasmid or with pLHL4. All transfections were performed using calcium phosphate as previously described (15).

20

25

β -galactosidase assay. Seventeen days following transfection with pCAR, β -galactosidase assays were performed using 20 μ g of protein in 45 mM 2-mercaptoethanol, 1mM MgCl₂, 0.1 M NaPO₄, and 0.6 mg/ml Chlorophenol red- β -D-galatopyranoside (CPRG, Boehringer Mannheim). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na₂CO₃, and analyzed by spectrophotometry at 576 nm (16). For *in situ* β -galactosidase staining, cells were fixed in 1% glutaraldehyde in PBS and incubated in 0.15 M NaCl, 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.2% X-Gal for 2 hours at 37°C.

30

EXAMPLE 2: hPMS2-134 Causes a Defect in MMR Activity

The most likely explanation for the differences in β -galactosidase activity between *PMS2-WT* and *PMS2-134* transfected cells was that the *PMS2-134* protein disturbed MMR activity, resulting in a higher frequency of mutation within the pCAR-OF reporter and re-establishing the ORF. To directly test the hypothesis that MMR was altered, we employed a biochemical assay for MMR with the individual clones described in Fig. 4. Nuclear extracts were prepared from the clones and incubated with heteroduplex substrates containing either a /CA\ insertion-deletion or a G/T mismatch under conditions described previously. The /CA\ and G/T heteroduplexes were used to test repair from the 3' and 5' directions, respectively. There was a dramatic difference between the *PMS2-134* expressing clones and the other clones in these assays (Table 2A). While all clones repaired substrates from the 3' direction (/CA\ heteroduplex), cells expressing the *PMS2-134* polypeptide had very little 5' repair activity. A similar directional defect in mismatch repair was evident with pooled clones resulting from *PMS2-134* transfection, or when the heteroduplex contained a 2-4 base pair loop, examples of which are shown in Table 2B. A small decrease in MMR activity was observed in the 3' /CA\ *PMS2-WT* repair assays, perhaps a result of interference in the biochemical assays by overexpression of the *PMS2* protein. No significant activity was caused by *PMS2-WT* in the *in situ* β -galactosidase assays (Fig. 3; Table 1), a result more likely to reflect the *in vivo* condition.

- 18 -

Table 2. Mismatch repair activity of nuclear extracts from SH clones (A) or pooled cultures (B). The extracts were tested for MMR activity with 24 fmol of heteroduplex. *These data represent similar results derived from greater than five independent experiments.

5

A. SH clones***Repaired substrate (fmol/15 min)**

3' /CA\ 5' G/T

10

Cell Line**PMS2-NOT**

clone A	10.2	3.5
clone B	12.7	2.9
clone C	13.5	5.5

15

PMS2-WT

clone A	2.8	2.2
clone B	5.7	4.8
clone C	4.7	2.9

20

PMS2-134

clone A	2.5	0.0
clone B	2.4	0.0
clone C	5.0	0.5

25

B. Pooled cultures

30

Repaired substrate (fmol/15 min)

3'G/T 5'G/T 3'CTG\ 5'CTG\

Cell Line

35

PMS2-NOT 2.07 +/- 0.09 2.37 +/- 0.37 3.45 +/- 1.35 2.77 +/- 1.37

40

PMS2-WT 1.65 +/- 0.94 1.86 +/- 0.57 1.13 +/- 0.23 1.23 +/- 0.65

PMS2-134 0.14 +/- 0.2 0.0 +/- 0.0 1.31 +/- 0.66 0.0 +/- 0.0

- 19 -

Western blots. Equal number of cells were lysed directly in lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-12% Tris-glycine gels (for analysis of full-length hPMS2) or 4-20% Tris-glycine gels (for analysis of hPMS2-134). Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a polyclonal antibody generated against residues 2 - 20 of hPMS2 (Santa Cruz Biotechnology, Inc.) and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody, using chemiluminescence for detection (Pierce).

In vitro translation. Linear DNA fragments containing *hPMS2* and *hMLH1* cDNA sequences were prepared by PCR, incorporating sequences for *in vitro* transcription and translation in the sense primer. A full-length *hMLH1* fragment was prepared using the sense primer 5'-ggatcctaatacgaactcactatagggaga ccaccatgtcggtcggtggcaggg-3' (codons 1-6) and the antisense primer 5'-taagtcttaagtgtaccaac-3' (located in the 3' untranslated region, nt 2411-2433), using a wild-type *hMLH1* cDNA clone as template. A full-length *hPMS2* fragment was prepared with the sense primer 5'-ggatcctaatacgaactcactatagggagaccaccatggaaacaattgcctgcgg-3' (codons 1- 6) and the antisense primer 5'-aggtagtgaagactctgtc-3' (located in 3' untranslated region, nt 2670-2690) using a cloned *hPMS2* cDNA as template. A fragment encoding the amino-terminal 134 amino acids of hPMS2 was prepared using the same sense primer and the antisense primer 5'-agtcgaggttccaacc.tcg-3'. A fragment containing codons 135 - 862 of *hPMS135* was generated using the sense primer 5'-ggatcctaatacgaactcactatagggagaccaccatgtttgatcacaatgg-3' (codons 135-141) and the same antisense primer as that used for the full-length

- 20 -

5

hPMS2 protein. These fragments were used to produce proteins via the coupled transcription-translation system (Promega). The reactions were supplemented with 35 S-labelled methionine or unlabelled methionine, as indicated in the text. The PMS135 and hMLH1 proteins could not be simultaneously radiolabelled and immunoprecipitated because of their similar molecular weights precluded resolution. Lower molecular weight bands are presumed to be degradation products and/or polypeptides translated from alternative internal methionines.

10

Immunoprecipitation. Immunoprecipitations were performed on *in vitro* translated proteins by mixing the translation reactions with 1 μ g of the MLH1 specific monoclonal antibody (mAB) MLH14 (Oncogene Science, Inc.), a polyclonal antibody generated to codons 2 - 20 of hPMS2 described above, or a polyclonal antibody generated to codons 843-862 of hPMS2 (Santa Cruz Biotechnology, Inc.) in 400 μ l of EBC buffer (50 mM Tris, pH 7.5, 0.1 M NaCl, 0.5% NP40). After incubation for 1 hr at 4°C, protein A sepharose (Sigma) was added to a final concentration of 10% and reactions were incubated at 4°C for 1 hour. Proteins bound to protein A were washed five times in EBC and separated by electrophoresis on 4-20% Tris-glycine gels, which were then dried and autoradiographed.

15

20

Biochemical assays for mismatch repair. MMR activity in nuclear extracts was performed as described, using 24 fmol of substrate (12,25). Complementation assays were done by adding ~ 100 ng of purified MutL α or MutS α components to 100 μ g of nuclear extract, adjusting the final KCl concentration to 100 mM (4,10,30). The substrates used in these experiments contain a strand break 181 nucleotides 5' or 125 nucleotides 3' to the mismatch. Values represent experiments performed at least in duplicate.

25

30

- 21 -

EXAMPLE 3: Carboxy Terminus of hPMS2 Mediates Interaction between hPMS2 and hMLH1

To elucidate the mechanism by which hPMS2-134 affected MMR, we analyzed the interaction between hPMS2 and hMLH1. Previous studies have shown that these two proteins dimerize to form a functionally active complex (12, 28). Proteins were synthesized *in vitro* using reticulocyte lysates, employing RNA generated from cloned templates. The full-length hMLH1 and hPMS2 proteins bound to each other and were co-precipitated with antibodies to either protein, as expected (data not shown). To determine the domain of hPMS2 which bound to hMLH1, the amino terminus (codons 1 - 134), containing the most highly conserved domain among mutL proteins (19,24), and the carboxyl terminus (codons 135 - 862) were separately cloned and proteins produced *in vitro* in coupled transcription-translation reactions. When a 35 S-labelled, full length hMLH1 protein (Fig. 5A, lane 5) was mixed with the unlabelled carboxyl terminal hPMS2 polypeptide, a monoclonal antibody (mAb) to the carboxyl terminus of hPMS2 efficiently immunoprecipitated the labeled hMLH1 protein (lane 1). No hMLH1 protein was precipitated in the absence of hPMS2 (lane 2). Conversely, when the 35 S-labelled carboxyl-terminus of hPMS2 (lane 3) was incubated with unlabelled, full-length hMLH1 protein, an anti-hMLH1 mAb precipitated the hPMS2 polypeptide (lane 4). In the absence of the unlabelled hMLH1 protein, no hPMS2 protein was precipitated by this mAb (lane 6). The same antibody failed to immunoprecipitate the amino-terminus of hPMS2 (amino acids 1-134) when mixed with unlabelled MLH1 protein (Fig. 5B, lane 1). This finding was corroborated by the converse experiment in which radiolabelled hPMS2-134 (Fig. 5C, lane 1) was unable to coprecipitate radiolabelled MLH1 when precipitations were done using an N-terminal hPMS2 antibody (Fig. 5C, lane 2) while this antibody was shown to be able to coprecipitate MLH1 when mixed with wild-type hPMS2 (Fig. 5C, lane 4).

- 22 -

5

10

15

20

25

30

The initial steps of MMR are dependent on two protein complexes, called MutS α and MutL α (14). As the amino terminus of hPMS2 did not mediate binding of hPMS2 to hMLH1, it was of interest to determine whether it might instead mediate the interaction between the MutL α complex (composed of hMLH1 and hPMS2, ref. 12) and the MutS α complex (composed of MSH2 and GTBP, ref. 4). Because previous studies have demonstrated that MSH2 and the MutL α components do not associate in solution (28), we were unable to assay for direct hPMS2-134:MutS α interaction. We therefore used a different approach to address this issue, and attempted to complement nuclear extracts from the various SH cell lines with MutS α or MutL α . If the truncated protein present in the PMS2-134 expressing SH cells was binding to MutS α and lowering its effective concentration in the extract, then adding intact MutS α should rescue the MMR defect in such extracts. Purified MutS α added to such extracts had no effect (Fig. 6). In contrast, addition of intact MutL α to the extract completely restored directional repair to the extracts from PMS2-134 cells (Fig. 6).

The results described above lead to several conclusions. First, expression of the amino-terminus of hPMS2 results in an increase in microsatellite instability, consistent with a replication error (RER) phenotype. That this elevated microsatellite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells. Interestingly, the expression of PMS2-134 resulted in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction (5). It is known from previous studies in both prokaryotes and eukaryotes that the separate enzymatic components mediate repair from the two different directions. Our results, in combination with

- 23 -

those of Drummond *et al.*, strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also demonstrate that a defect in directional MMR is sufficient to produce a RER+ phenotype.

We anticipated that the dominant negative function of the PMS2-134 polypeptide resulted from its binding to MLH1 and consequent inhibition of MutL α function. This hypothesis was based in part on the fact that the most highly conserved domain of the PMS2 gene is located in its amino terminus, and the only known biochemical partner for PMS2 is MLH1. Our binding studies revealed, however, that the carboxyl terminus of PMS2, rather than the highly conserved amino terminus, actually mediated binding to MLH1. This result is consistent with those recently obtained in *S. cerevisiae*, in which the MLH1-interacting domain of PMS1 (the yeast homolog of human PMS2) was localized to its carboxyl-terminus (23). Our add-back experiments additionally showed that the hPMS2-134 mutant was not likely to mediate an interaction with the MutS α complex (Fig. 6). The best explanation at present to explain the various observations made here is that the hPMS2-134 polypeptide does not inhibit the initial steps in MMR, but rather interacts with and inhibits a downstream component of the pathway, perhaps a nuclease required for repair from the 5' direction.

The demonstration that the hPMS2-134 mutation can confer a dominant negative MMR defect to transfected cells helps to explain the phenotype of the kindred in which this mutant was discovered. Three individuals from this kindred were found to carry the mutation, a father and his two children. Both children exhibited microsatellite instability in their normal tissues and both developed tumors at an early age, while the father had no evidence of microsatellite instability in his normal cells and was completely healthy at age 35. The only difference known to us with respect to the MMR genes in this family is that the father's mutant allele was

- 24 -

5

10

expressed at lower levels than the wild-type allele as assessed by sequencing of reverse transcriptase-PCR products of RNA from lymphocytes. The children expressed both alleles at approximately equal levels (Parsons *et al.* and unpublished observations). We suspect that the dominant negative attribute of the *hPMS2-134* mutant will only be manifest when it is present at sufficient concentrations (at least equimolar), thus explaining the absence of MMR deficiency in the father. The reason for the differential expression of the *hPMS2-134* allele in this kindred is not clear, though imprinting is a possibility. Hopefully, the ascertainment of additional, larger kindreds with such mutations will facilitate the investigation of this issue.

- 25 -

References

1. Baker S.M., Bronner, C.E., Zhang, L., Plug, A.W., Robatez, M., Warren, G., Elliott, E.A., Yu, J., Ashley, T., Arnheim, N., Bradley, N., Flavell, R.A., and Liskay, R.M. 1995. Male defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* 82:309-319.

5

2. Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag, R.J., Godwin, A., R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. 1994. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* 368:258-261.

10

3. de Wind N., Dekker, M., Berns, A., Radman, M., and Riele, H.T. 1995. Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* 82:321-300.

15

4. Drummond, J.T., Li, G.M., Longley, M.J., and Modrich, P. 1995. Isolation of an hMSH2-p160 heterodimer that restores mismatch repair to tumor cells. *Science* 268:1909-1912.

20

5. Drummond, J.T., Anthoney, A., Brown, R., and Modrich, P. 1996. Cisplatin and adriamycin resistance are associated with MutL α and mismatch repair deficiency in an ovarian tumor cell line. *J.Biol.Chem.* 271:9645-19648.

25

6. Edelmann, W., Cohen, P.E., Kane, M., Lau, K., Morrow, B., Bennett, S., Umar, A., Kunkel, T., Cattoretti, G., Chagnatti, R., Pollard, J.W., Kolodner, R.D., and Kucherlapati, R. 1996. Meiotic pachytene arrest in MLH1-deficient mice. *Cell* 85:1125-1134.

30

- 26 -

7. Fishel, R., Lescoe, M., Rao, M.R.S., Copeland, N.J., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. 1993. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 7:1027-1038.

5

8. Green, S., Issemann, I., and Sheer, E. 1988. A versatile *in vivo* eucaryotic expression vector for protein engineering. *Nuc. Acid Res.* 16:369.

10

9. Hamilton, S.R., Liu, B., Parsons, R.E., Papadopoulos, N., Jen, J., Powell, S.M., Krush, A.J., Berk, T., Cohen, Z., tetu, B., Kinzler, K.W., and Vogelstein, B. 1995. The molecular basis of Turcot's syndrome. *N. Eng. J. Med.* 332:839-847.

15

10. Holmes, J., Clark, S., and Modrich, P. Strand specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. (1990). *Proc. Natl. Acad. Sci. USA* 87:5837- 5841.

20

11. Leach, F.S., Nicolaides, N.C, Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, J.A., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. 1993. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. *Cell* 75:1215-1225.

25

12. Li, G.-M., and Modrich, P. 1995. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human *mutL* homologs. *Proc. Natl. Acad. Sci. USA* 92:1950-1954.

30

- 27 -

13. Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat. Med.* 2:169-174.

5

14. Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. *Science* 266:1959-1960.

10

15. Nicolaides, N.C., Gualdi, R., Casadevall, C., Manzella, L., and Calabretta, B. 1991. Positive autoregulation of *c-myb* expression via MYB binding in the 5' flanking region of the human *c-myb* gene. *Mol. Cell. Biol.* 11:6166-6176.

15

16. Nicolaides, N.C., Correa, I., Casadevall, C., Travali, S., Soprano, K.J., and Calabretta, B. 1992. The Jun family members, c-JUN and JUND, transactivate the human *c-myb* promoter via an Ap1 like element. *J. Biol. Chem.* 267, 19665-19672.

20

17. Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutations of two PMS homologs in hereditary nonpolyposis colon cancer. *Nature* 371: 75-80.

25

18. Nicolaides N.C., Kinzler, K.W., and Vogelstein, B. 1995. Analysis of the 5' region of PMS2 reveals heterogenous transcripts and a novel overlapping gene. *Genomics* 29:329-334.

30

- 28 -

19. Nicolaides, N.C., Carter, K.C., Shell, B.K., Papadopoulos, N., Vogelstein, B., and Kinzler, K.W. 1995. Genomic organization of the human PMS2 gene family. *Genomics* 30:195-206.

5 20. Nicolaides, N.C., Palombo, F., Kinzler, K.W., Vogelstein, B., and Jiricny, J. 1996. Molecular cloning of the N-terminus of GTBP. *Genomics* 31:395-397.

10 21. Palombo, F., Hughes, M., Jiricny, J., Truong, O., Hsuan, J. 1994. Mismatch repair and cancer. *Nature* 36:417.

15 22. Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M.A., Truong, O., Hsuan, J.J., and Jiricny, J. 1995. GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. *Science* 268:1912-1914.

20 23. Pang, Q., Prolla, T.A., and Liskay, R.M. 1997. Functional domains of the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA mismatch repair proteins and their relevance to human hereditary nonpolyposis colorectal cancer-associated mutations. *Mol. Cell. Biol.* 17:4465-4473.

25 24. Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutation of a mutL homolog is associated with hereditary colon cancer. *Science* 263:1625-1629.

30 25. Parsons, R., Li, G.M., Longley, M.J., Fang, W.H., Papadopolous, N., Jen, J., de la Chapelle, A., Kinzler, K.W., Vogelstein, B., and Modrich, P.

- 29 -

1993. Hypermutability and mismatch repair deficiency in RER⁺ tumor cells. *Cell* 75:1227-1236.

5 26. Parsons, R., Li, G.M., Longley, M., Modrich, P., Liu, B., Berk, T., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. 1995. Mismatch repair deficiency in phenotypically normal human cells. *Science* 268:738-740.

10 27. Perucho, M. 1996. Cancer of the microsatellite mutator phenotype. *Biol Chem* 377:675-684.

15 28. Prolla, T.A., Pang, Q., Alani, E., Kolodner, R.A., and Liskay, R.M. 1994. MLH1, PMS1, and MSH2 Interaction during the initiation of DNA mismatch repair in yeast. *Science* 264:1091-1093.

20 29. Strand, M., Prolla, T.A., Liskay, R.M., and Petes, T.D. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. 1993. *Nature* 365:274-276.

30. Su, S.S., Lahue, R.S., Au, K.G., and Modrich, P. 1988. Mispair specificity of methyl directed DNA mismatch corrections in vitro. *J. Biol. Chem.* 263:6829-6835.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT:
(A) NAME: THE JOHNS HOPKINS UNIVERSITY
(B) STREET: 720 Rutland Avenue,
(C) CITY: Baltimore, Maryland
(E) COUNTRY: USA
(F) POSTAL CODE: 21205

(ii) TITLE OF THE INVENTION: A Method for Generating Hypermutable Organisms

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
John H. Woodley
Sim & McBurney
330 University Avenue, 6th Floor
Toronto, Canada M5G 1R7

(v) COMPUTER READABLE FORM:
(A) COMPUTER: IBM PC compatible
(B) OPERATING SYSTEM: PC-DOS/MS-DOS
(C) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 2,240,609
(B) FILING DATE: August 11, 1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER : US 09/059,461
(B) FILING DATE: April 14, 1998
(C) CLASSIFICATION:

(viii) PATENT AGENT INFORMATION:
(A) NAME: John H. Woodley
(B) REFERENCE NUMBER: JHW 19-1126

(2) INFORMATION FOR SEQ. ID NO. 1.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2771 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY : linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 1

cgaggcggat cgggtgtgc atcc atg gag cga gct gag agc tcg agt aca	51
gaa cct gct aag gcc atc aaa cct att gat cgg aag tca gtc cat cag	99
att tgc tct ggg cag gtg gta ctg agt cta agc act gcg gta aag gag	147
tta gta gaa aac agt ctg gat gct ggt gcc act aat att gat cta aag	195
ctt aag gac tat gga gtg gat ctt att gaa gtt tca gac aat gga tgt	243
ggg gta gaa gaa aac ttc gaa ggc tta act ctg aaa cat cac aca	291
tct aag att caa gag ttt gcc gac cta act cag gtt gaa act ttt ggc	339
ttt cgg ggg gaa gct ctg agc tca ctt tgt gca ctg agc gat gtc acc	387
att tct acc tgc cac gca tcg gcg aag gtt gga act cga ctg atg ttt	435
gat cac aat ggg aaa att atc cag aaa acc ccc tac ccc cgc ccc aga	483
ggg acc aca gtc agc gtg cag cag tta ttt tcc aca cta cct gtg cgc	531
cat aag gaa ttt caa agg aat att aag aag gag tat gcc aaa atg gtc	579
cag gtc tta cat gca tac tgt atc att tca gca ggc atc cgt gta agt	627
tgc acc aat cag ctt gga caa gga aaa cga cag cct gtg gta tgc aca	675
ggt gga agc ccc agc ata aag gaa aat atc ggc tct gtg ttt ggg cag	723
aag cag ttg caa agc ctc att cct ttt gtt cag ctg ccc cct agt gac	771
tcc gtg tgt gaa gag tac ggt ttg agc tgt tcg gat gct ctg cat aat	819
ctt ttt tac atc tca ggt ttc att tca caa tgc acg cat gga gtt gga	867
agg agt tca aca gac aga cag ttt ttc ttt atc aac cgg cgg cct tgt	915
gac cca gca aag gtc tgc aga ctc gtg aat gag gtc tac cac atg tat	963
aat cga cac cag tat cca ttt gtt ctt aac att tct gtt gat tca	1011
gaa tgc gtt gat atc aat gtt act cca gat aaa agg caa att ttg cta	1059
caa gag gaa aag ctt ttg ttg gca gtt tta aag acc tct ttg ata gga	1107
atg ttt gat agt gat gtc aac aag cta aat gtc agt cag cag cca ctg	1155
ctg gat gtt gaa ggt aac tta ata aaa atg cat gca gcg gat ttg gaa	1203
aag ccc atg gta gaa aag cag gat caa tcc cct tca tta agg act gga	1251
gaa gaa aaa aaa gac gtg tcc att tcc aga ctg cga gag gcc ttt tct	1299
ctt cgt cac aca aca gag aac aag cct cac agc cca aag act cca gaa	1347

cca aga agg agc cct cta gga cag aaa agg ggt atg ctg tct tct agc	1395
act tca ggt gcc atc tct gac aaa ggc gtc ctg aga cct cag aaa gag	1443
gca gtg agt tcc agt cac gga ccc agt gac cct acg gac aga gcg gag	1491
gtg gag aag gac tcg ggg cac ggc agc act tcc gtg gat tct gag ggg	1539
ttc agc atc cca gac acg ggc agt cac tgc agc agc gag tat gcg gcc	1587
agc tcc cca ggg gac agg ggc tcg cag gaa cat gtg gac tct cag gag	1635
aaa gcg cct gaa act gac gac tct ttt tca gat gtg gac tgc cat tca	1683
aac cag gaa gat acc gga tgt aaa ttt cga gtt ttg cct cag cca act	1731
aat ctc gca acc cca aac aca aag cgt ttt aaa aaa gaa gaa att ctt	1779
tcc agt tct gac att tgt caa aag tta gta aat act cag gac atg tca	1827
gcc tct cag gtt gat gta gct gtg aaa att aat aag aaa gtt gtg ccc	1875
ctg gac ttt tct atg agt tct tta gct aaa cga ata aag cag tta cat	1923
cat gaa gca cag caa agt gaa ggg gaa cag aat tac agg aag ttt agg	1971
gca aag att tgt cct gga gaa aat caa gca gcc gaa gat gaa cta aga	2019
aaa gag ata agt aaa acg atg ttt gca gaa atg gaa atc att ggt cag	2067
ttt aac ctg gga ttt ata ata acc aaa ctg aat gag gat atc ttc ata	2115
gtg gac cag cat gcc acg gac gag aag tat aac ttc gag atg ctg cag	2163
cag cac acc gtg ctc cag ggg cag agg ctc ata gca cct cag act ctc	2211
aac tta act gct gtt aat gaa gct gtt ctg ata gaa aat ctg gaa ata	2259
ttt aga aag aat ggc ttt gat ttt gtt atc gat gaa aat gct cca gtc	2307
act gaa agg gct aaa ctg att tcc ttg cca act agt aaa aac tgg acc	2355
ttc gga ccc cag gac gtc gat gaa ctg atc ttc atg ctg agc gac agc	2403
cct ggg gtc atg tgc cgg cct tcc cga gtc aag cag atg ttt gcc tcc	2451
aga gcc tgc cgg aag tcg gtg atg att ggg act gct ctt aac aca agc	2499
gag atg aag aaa ctg atc acc cac atg ggg gag atg gac cac ccc tgg	2547
aac tgt ccc cat gga agg cca acc atg aga cac atc gcc aac ctg ggt	2595
gtc att tct cag aac tgaccgtagt cactgtatgg aataatttgt tttatcgac	2650
attttatgt tttgaaagac agagtcttca ctaacctttt ttgtttaaa atgaaacctg	2710
ctacttaaaa aaaatacaca tcacacccat taaaagtga tcttggaaac ctttcaaacc	2770
c	2771

(2) INFORMATION FOR SEQUENCE ID. NO. 2

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 862 Amino Acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS : single
- (D) TOPOLOGY : linear

(ii) MOLECULAR TYPE: Protein

(xi) SEQUENCE DESCRIPTION : SEQ. ID. NO. 2

Met	Glu	Arg	Ala	Glu	Ser	Ser	Ser	Thr	Glu	Pro	Ala	Lys	Ala	Ile	Lys
1	5							10				15			
Pro	Ile	Asp	Arg	Lys	Ser	Val	His	Gln	Ile	Cys	Ser	Gly	Gln	Val	Val
	20					25						30			
Leu	Ser	Leu	Ser	Thr	Ala	Val	Lys	Glu	Leu	Val	Glu	Asn	Ser	Leu	Asp
	35					40						45			
Ala	Gly	Ala	Thr	Asn	Ile	Asp	Leu	Lys	Leu	Lys	Asp	Tyr	Gly	Val	Asp
	50					55						60			
Leu	Ile	Glu	Val	Ser	Asp	Asn	Gly	Cys	Gly	Val	Glu	Glu	Asn	Phe	
	65				70				75					80	
Glu	Gly	Leu	Thr	Leu	Lys	His	His	Thr	Ser	Lys	Ile	Gln	Glu	Phe	Ala
		85				90			95						
Asp	Leu	Thr	Gln	Val	Glu	Thr	Phe	Gly	Phe	Arg	Gly	Glu	Ala	Leu	Ser
	100					105						110			
Ser	Leu	Cys	Ala	Leu	Ser	Asp	Val	Thr	Ile	Ser	Thr	Cys	His	Ala	Ser
	115					120			125						
Ala	Lys	Val	Gly	Thr	Arg	Leu	Met	Phe	Asp	His	Asn	Gly	Lys	Ile	Ile
	130					135			140						
Gln	Lys	Thr	Pro	Tyr	Pro	Arg	Pro	Arg	Gly	Thr	Thr	Val	Ser	Val	Gln
	145					150			155					160	
Gln	Leu	Phe	Ser	Thr	Leu	Pro	Val	Arg	His	Lys	Glu	Phe	Gln	Arg	Asn
		165					170					175			
Ile	Lys	Lys	Glu	Tyr	Ala	Lys	Met	Val	Gln	Val	Leu	His	Ala	Tyr	Cys
		180					185					190			
Ile	Ile	Ser	Ala	Gly	Ile	Arg	Val	Ser	Cys	Thr	Asn	Gln	Leu	Gly	Gln
	195					200					205				
Gly	Lys	Arg	Gln	Pro	Val	Val	Cys	Thr	Gly	Gly	Ser	Pro	Ser	Ile	Lys
	210					215			220						
Glu	Asn	Ile	Gly	Ser	Val	Phe	Gly	Gln	Lys	Gln	Leu	Gln	Ser	Leu	Ile
	225					230			235					240	
Pro	Phe	Val	Gln	Leu	Pro	Pro	Ser	Asp	Ser	Val	Cys	Glu	Glu	Tyr	Gly
		245					250						255		
Leu	Ser	Cys	Ser	Asp	Ala	Leu	His	Asn	Leu	Phe	Tyr	Ile	Ser	Gly	Phe
	260					265						270			
Ile	Ser	Gln	Cys	Thr	His	Gly	Val	Gly	Arg	Ser	Ser	Thr	Asp	Arg	Gln
	275						280					285			
Phe	Phe	Phe	Ile	Asn	Arg	Arg	Pro	Cys	Asp	Pro	Ala	Lys	Val	Cys	Arg
	290					295						300			
Leu	Val	Asn	Glu	Val	Tyr	His	Met	Tyr	Asn	Arg	His	Gln	Tyr	Pro	Phe
	305					310			315					320	
Val	Val	Leu	Asn	Ile	Ser	Val	Asp	Ser	Glu	Cys	Val	Asp	Ile	Asn	Val
		325					330					335			

Thr Pro Asp Lys Arg Gln Ile Leu Leu Gln Glu Glu Lys Leu Leu Leu
 340 345 350
 Ala Val Leu Lys Thr Ser Leu Ile Gly Met Phe Asp Ser Asp Val Asn
 355 360 365
 Lys Leu Asn Val Ser Gln Gln Pro Leu Leu Asp Val Glu Gly Asn Leu
 370 375 380
 Ile Lys Met His Ala Ala Asp Leu Glu Lys Pro Met Val Glu Lys Gln
 385 390 395 400
 Asp Gln Ser Pro Ser Leu Arg Thr Gly Glu Glu Lys Lys Asp Val Ser
 405 410 415
 Ile Ser Arg Leu Arg Glu Ala Phe Ser Leu Arg His Thr Thr Glu Asn
 420 425 430
 Lys Pro His Ser Pro Lys Thr Pro Glu Pro Arg Arg Ser Pro Leu Gly
 435 440 445
 Gln Lys Arg Gly Met Leu Ser Ser Ser Thr Ser Gly Ala Ile Ser Asp
 450 455 460
 Lys Gly Val Leu Arg Pro Gln Lys Glu Ala Val Ser Ser Ser His Gly
 465 470 475 480
 Pro Ser Asp Pro Thr Asp Arg Ala Glu Val Glu Lys Asp Ser Gly His
 485 490 495
 Gly Ser Thr Ser Val Asp Ser Glu Gly Phe Ser Ile Pro Asp Thr Gly
 500 505 510
 Ser His Cys Ser Ser Glu Tyr Ala Ala Ser Ser Pro Gly Asp Arg Gly
 515 520 525
 Ser Gln Glu His Val Asp Ser Gln Glu Lys Ala Pro Glu Thr Asp Asp
 530 535 540
 Ser Phe Ser Asp Val Asp Cys His Ser Asn Gln Glu Asp Thr Gly Cys
 545 550 555 560
 Lys Phe Arg Val Leu Pro Gln Pro Thr Asn Leu Ala Thr Pro Asn Thr
 565 570 575
 Lys Arg Phe Lys Lys Glu Glu Ile Leu Ser Ser Asp Ile Cys Gln
 580 585 590
 Lys Leu Val Asn Thr Gln Asp Met Ser Ala Ser Gln Val Asp Val Ala
 595 600 605
 Val Lys Ile Asn Lys Lys Val Val Pro Leu Asp Phe Ser Met Ser Ser
 610 615 620
 Leu Ala Lys Arg Ile Lys Gln Leu His His Glu Ala Gln Gln Ser Glu
 625 630 635 640
 Gly Glu Gln Asn Tyr Arg Lys Phe Arg Ala Lys Ile Cys Pro Gly Glu
 645 650 655
 Asn Gln Ala Ala Glu Asp Glu Leu Arg Lys Glu Ile Ser Lys Thr Met
 660 665 670
 Phe Ala Glu Met Glu Ile Ile Gly Gln Phe Asn Leu Gly Phe Ile Ile
 675 680 685
 Thr Lys Leu Asn Glu Asp Ile Phe Ile Val Asp Gln His Ala Thr Asp
 690 695 700
 Glu Lys Tyr Asn Phe Glu Met Leu Gln Gln His Thr Val Leu Gln Gly
 705 710 715 720
 Gln Arg Leu Ile Ala Pro Gln Thr Leu Asn Leu Thr Ala Val Asn Glu
 725 730 735
 Ala Val Leu Ile Glu Asn Leu Glu Ile Phe Arg Lys Asn Gly Phe Asp
 740 745 750
 Phe Val Ile Asp Glu Asn Ala Pro Val Thr Glu Arg Ala Lys Leu Ile
 755 760 765
 Ser Leu Pro Thr Ser Lys Asn Trp Thr Phe Gly Pro Gln Asp Val Asp
 770 775 780
 Glu Leu Ile Phe Met Leu Ser Asp Ser Pro Gly Val Met Cys Arg Pro

785	790	795	800
Ser Arg Val Lys Gln Met Phe Ala Ser Arg Ala Cys Arg Lys Ser Val			
805	810	815	
Met Ile Gly Thr Ala Leu Asn Thr Ser Glu Met Lys Lys Leu Ile Thr			
820	825	830	
His Met Gly Glu Met Asp His Pro Trp Asn Cys Pro His Gly Arg Pro			
835	840	845	
Thr Met Arg His Ile Ala Asn Leu Gly Val Ile Ser Gln Asn			
850	855	860	

WE CLAIM:

5

1. A method for making a hypermutable cell, comprising the step of introducing into a mammalian cell a polynucleotide comprising a dominant negative allele of a mismatch repair gene, whereby the cell becomes hypermutable.

10

2. The method of claim 1 wherein the polynucleotide is introduced by transfection of a suspension of cells *in vitro*.

3. The method of claim 1 wherein the mismatch repair gene is *PMS2*.

4. The method of claim 1 wherein the mismatch repair gene is human *PMS2*.

5. The method of claim 1 wherein the mismatch repair gene is human *MLH1*.

15

6. The method of claim 1 wherein the mismatch repair gene is human *PMS1*.

7. The method of claim 1 wherein the mismatch repair gene is human *MSH2*.

8. The method of claim 4 wherein the allele comprises a truncation mutation.

9. The method of claim 4 wherein the allele comprises a truncation mutation at codon 134 as shown in SEQ ID NO: 1.

20

10. The method of claim 9 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.

11. The method of claim 1 wherein the polynucleotide is introduced into a fertilized egg of an animal.

25

12. The method of claim 11 wherein the fertilized egg is subsequently implanted into a pseudo-pregnant female whereby the fertilized egg develops into a mature transgenic animal.

13. The method of claim 12 wherein the mismatch repair gene is *PMS2*.

14. The method of claim 12 wherein the mismatch repair gene is human *PMS2*.

30

15. The method of claim 12 wherein the mismatch repair gene is human *MLH1*.

- 39 -

16. The method of claim 12 wherein the mismatch repair gene is human *PMS1*.
17. The method of claim 12 wherein the mismatch repair gene is human *MSH2*.
- 5 18. The method of claim 14 wherein the allele comprises a truncation mutation.
19. The method of claim 14 wherein the allele comprises a truncation mutation at codon 134 as shown in SEQ ID NO: 1.
- 10 20. The method of claim 19 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.
21. A homogeneous composition of cultured, hypermutable, mammalian cells which comprise a dominant negative allele of a mismatch repair gene.
22. The isolated hypermutable cell of claim 21 wherein the mismatch repair gene is *PMS2*.
- 15 23. The isolated hypermutable cell of claim 21 wherein the mismatch repair gene is human *PMS2*.
24. The isolated hypermutable cell of claim 21 wherein the mismatch repair gene is human *MLH1*.
- 25 20 25. The isolated hypermutable cell of claim 21 wherein the mismatch repair gene is human *PMS1*.
26. The isolated hypermutable cell of claim 21 wherein the mismatch repair gene is human *MSH2*.
27. The isolated hypermutable cell of claim 21 wherein the cells express a protein consisting of the first 133 amino acids of hPMS2.
28. A hypermutable transgenic mammal wherein at least 50% of the cells of the mammal comprise a dominant negative allele of a mismatch repair gene.
29. The hypermutable transgenic animal of claim 28 comprising a protein which consists of the first 133 amino acids of human PMS2.
30. A method for generating a mutation in a gene of interest comprising the steps of:

- 40 -

growing a mammalian cell comprising the gene of interest and a dominant negative allele of a mismatch repair gene, wherein the cell is hypermutable;

5 testing the cell to determine whether the gene of interest harbors a mutation.

31. The method of claim 30 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

32. The method of claim 30 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

10 33. The method of claim 30 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

34. The method of claim 30 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

15 35. The method of claim 30 wherein the mammalian cell is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a mammalian cell, whereby the cell becomes hypermutable.

36. The method of claim 35 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

20 37. The method of claim 35 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

38. The method of claim 35 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

25 39. The method of claim 35 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

40. A method for generating a mutation in a gene of interest comprising the steps of:

30 growing a mammal comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene;

- 41 -

testing the mammal to determine whether the gene of interest harbors a mutation.

41. The method of claim 40 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.
- 5 42. The method of claim 40 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.
43. The method of claim 40 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.
- 10 44. The method of claim 40 wherein the step of testing comprises analyzing the phenotype of the gene of interest.
45. The method of claim 40 wherein the mammal is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a mammal, whereby the mammal becomes hypermutable.
- 15 46. The method of claim 45 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.
47. The method of claim 45 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.
48. The method of claim 45 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.
- 20 49. The method of claim 45 wherein the step of testing comprises analyzing the phenotype of the gene of interest.
50. A hypermutable transgenic mammal made by the method of claim 45.
51. The transgenic mammal of claim 50 wherein the mammal is a primate.
- 25 52. The transgenic mammal of claim 50 wherein the mismatch repair gene is *PMS2*.
53. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *PMS2*.
54. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *MLH1*.

- 42 -

55. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *PMS1*.

56. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *MSH2*.

5 57. The transgenic mammal of claim 50 wherein the allele comprises a truncation mutation.

58. The transgenic mammal of claim 50 wherein the allele comprises a truncation mutation at codon 134 as shown in SEQ ID NO: 1.

10 59. The transgenic mammal of claim 58 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.

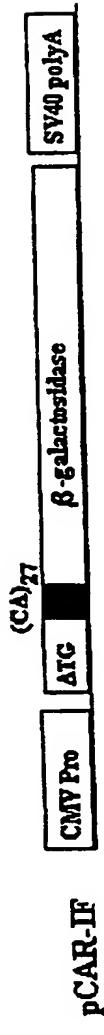
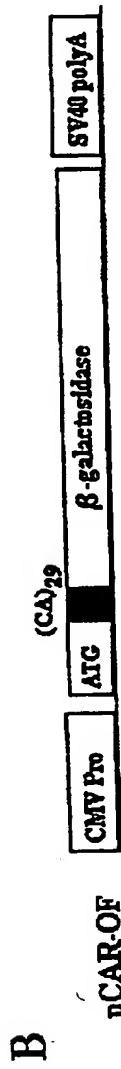
- 43 -

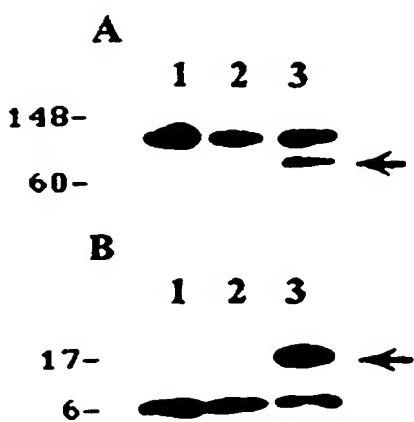
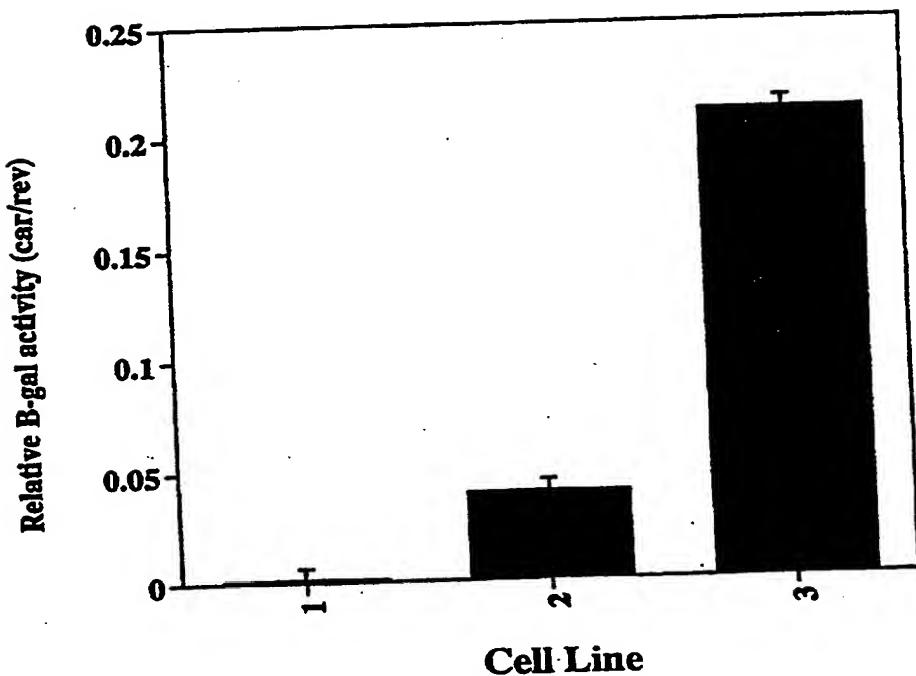
A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

ABSTRACT

5 Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation.

A



Nicolaides et.al.
Figure 2**C**

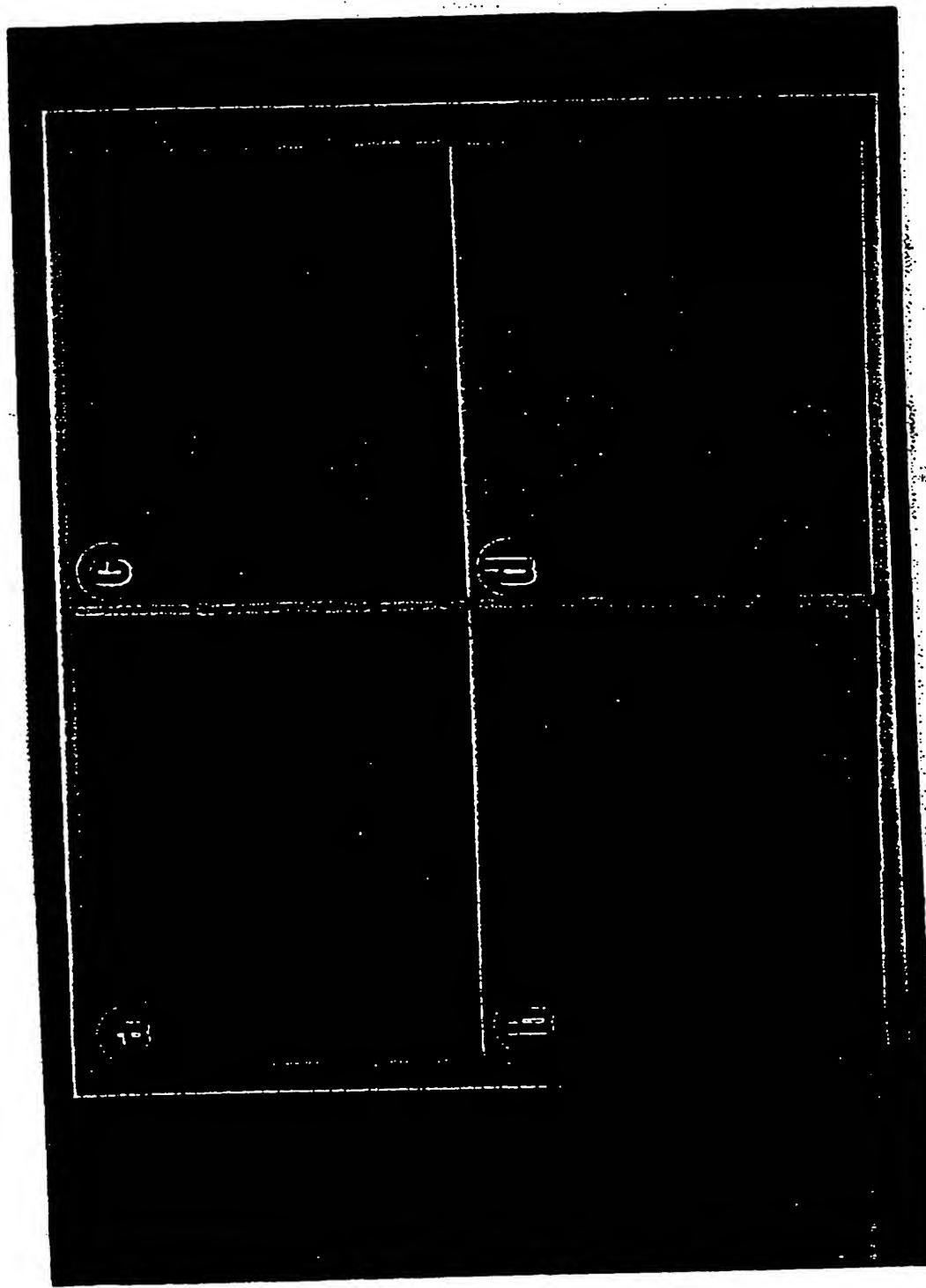


Fig 3

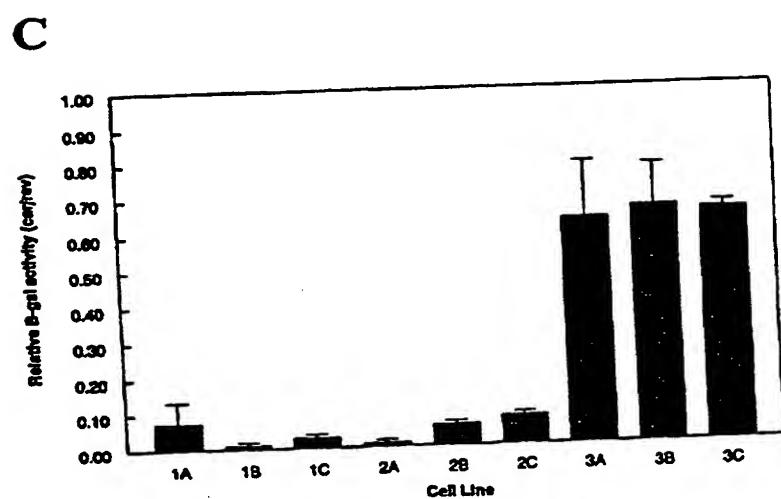
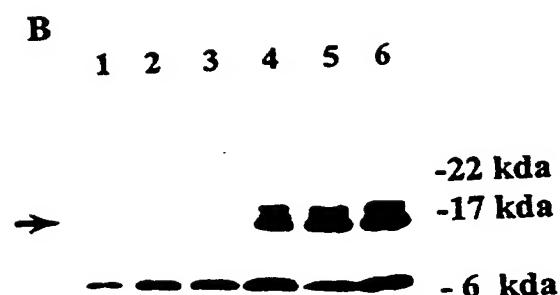
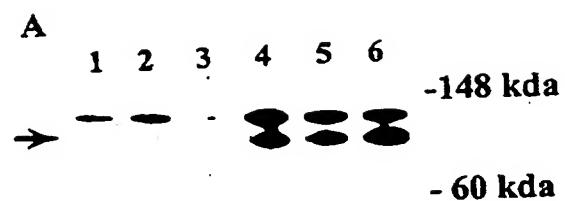


Fig. L!

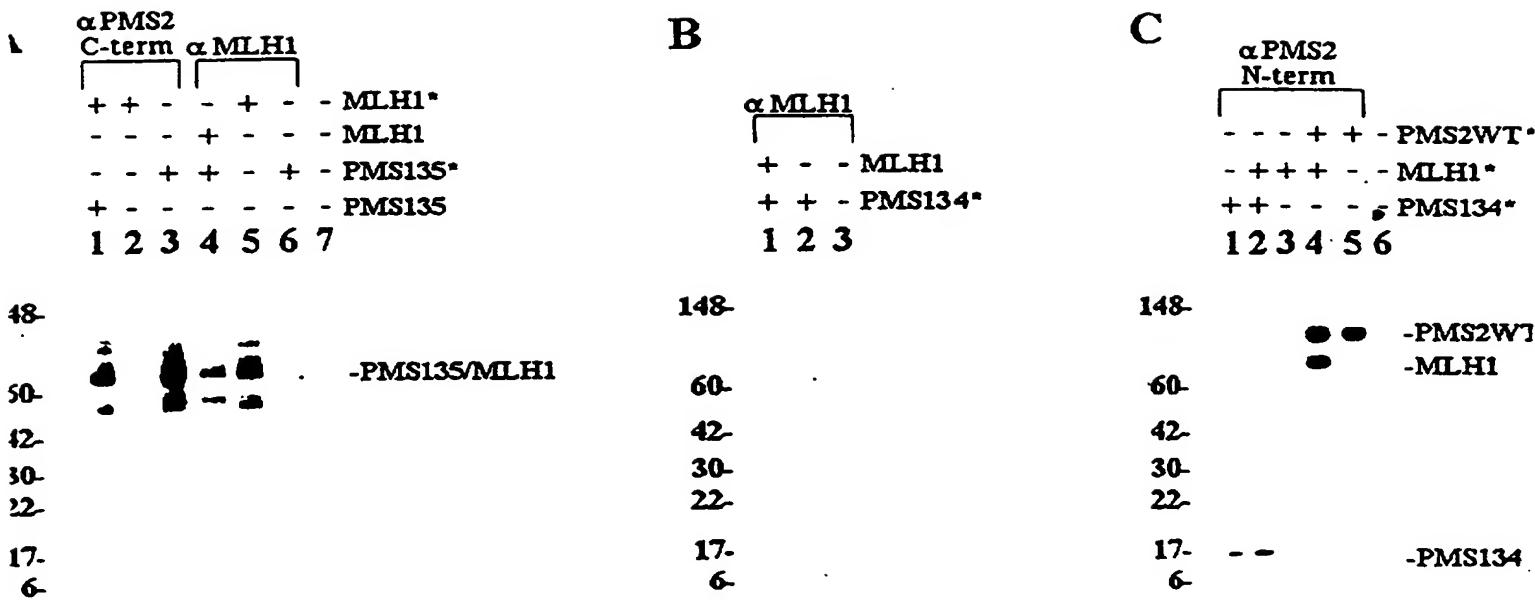
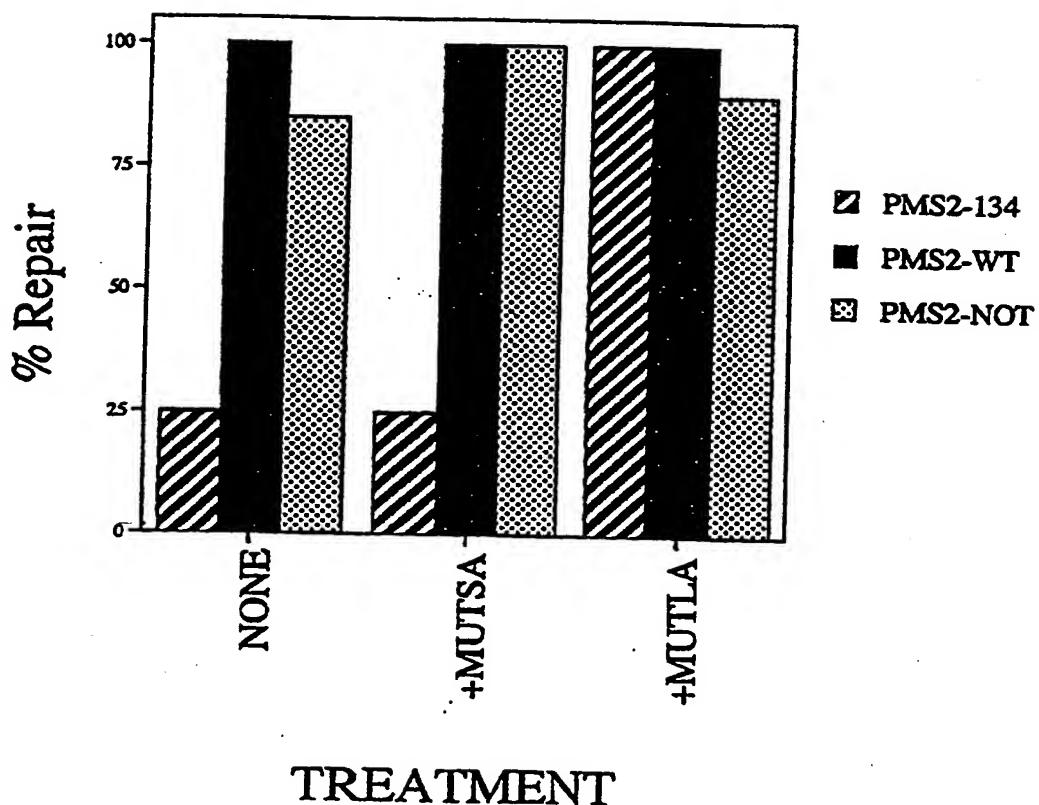


Fig.5

Nicolaides et.al.
Figure 6



A

PMSS1-NOT	ST40 P ₀	polymer	ST40 polyA
-----------	---------------------	---------	------------

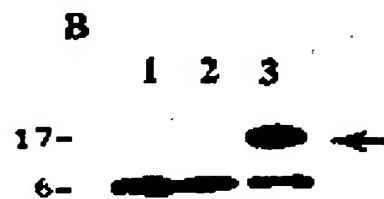
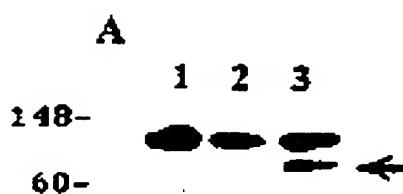
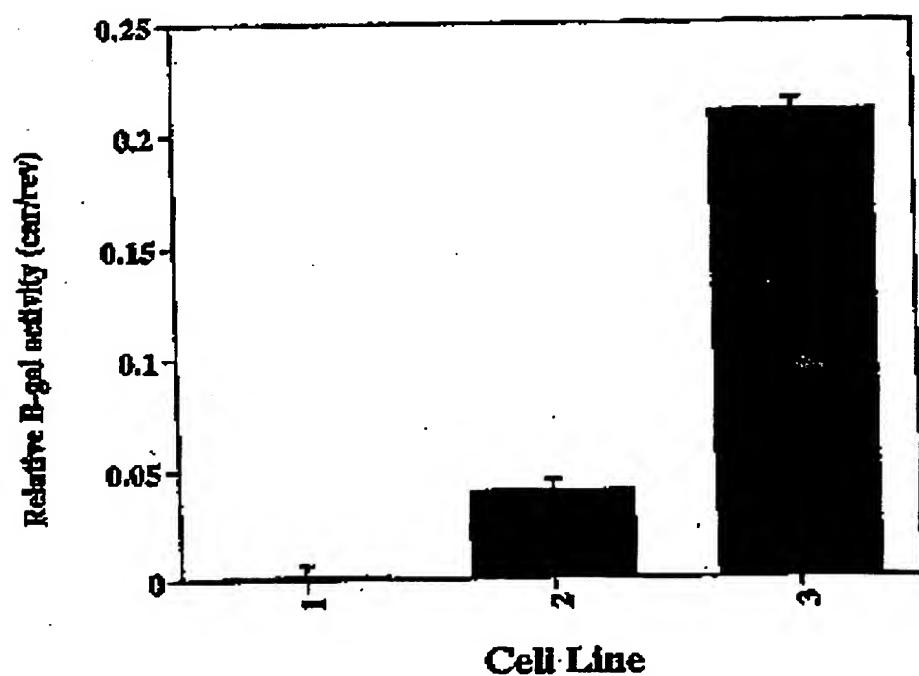
PMSS1-WT	ST40 P ₀	Impacted (60° sec)	ST40 polyA
----------	---------------------	--------------------	------------

PMSS2-134	ST40 P ₀	PMSS2 (60° sec)	ST40 polyA
-----------	---------------------	-----------------	------------

B

pCAR-OF	CMV P ₀	(CA) ₉	ARC	β-galactosidase	ST40 polyA
---------	--------------------	-------------------	-----	-----------------	------------

pCAR-IF	CMV P ₀	(CA) ₇	ARC	β-galactosidase	ST40 polyA
---------	--------------------	-------------------	-----	-----------------	------------

Nicolaides et al.
Figure 2**C**

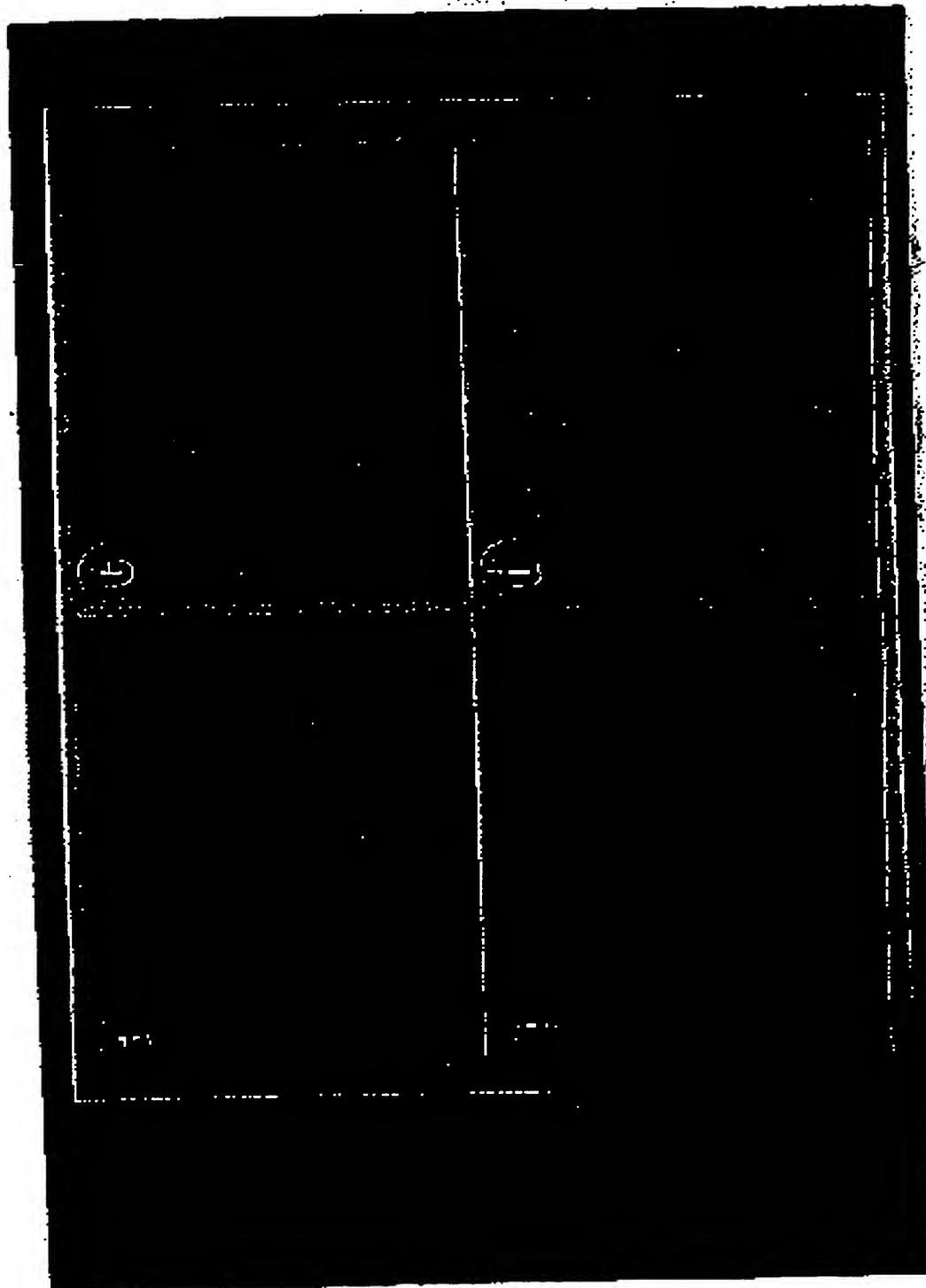


Fig 3

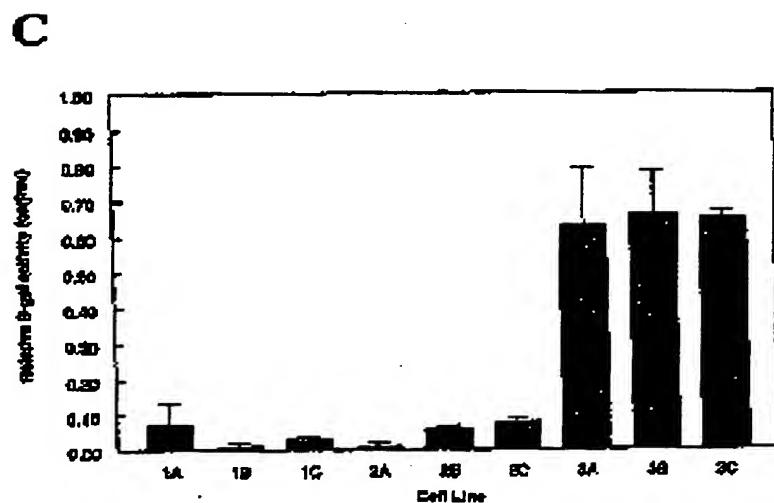
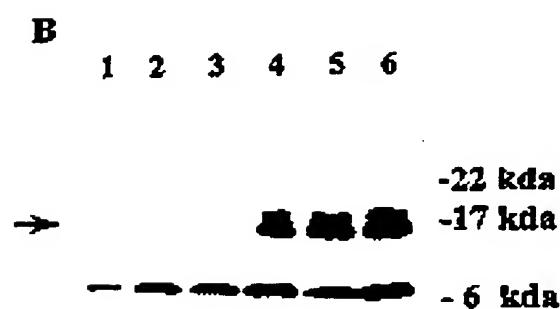
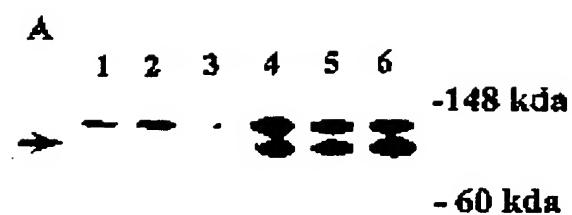


Fig 4

α PMS2
C-term α MLH1

	+	+	-	+	-	-	-	-	-	-
MLH1*	+	+	-	+	-	-	-	-	-	-
MLH1	-	-	-	+	-	-	-	-	-	-
PMS135*	-	-	+	+	-	+	-	-	-	-
PMS135	+	-	-	-	-	-	-	-	-	-

1 2 3 4 5 6 7

B

α MLH1

	+	-	-	-
MLH1	+	-	-	-
PMS134*	+	+	-	-

1 2 3

C

α PMS2
N-term

	-	-	+	+	-	-
PMS2WT*	-	-	+	+	-	-
MLH1*	-	+	+	+	-	-
PMS134*	+	+	-	-	-	-

1 2 3 4 5 6

43-



-PMS135/MLH1

148-

60.
42.
30.
22.
17.
6.

148-

60.
42.
30.
22.
17.
6.

-PMS2WT
-MLH1
-PMS134

Nicolaides et.al.
Figure 6